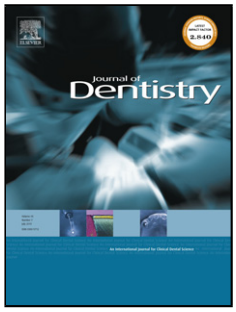

Title: Polymerisation, antibacterial and bioactivity properties of experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes

Authors: Felipe Weidenbach Degrazia, Bruna Genari, Vicente Castelo Branco Leitune, Rodrigo Alex Arthur, Santiago Arias Luxan, Susana Maria Werner Samuel, Fabrício Mezzomo Collares, Salvatore Sauro

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Manuscript Draft

Manuscript Number: JJOD-D-17-00749R1

Title: Polymerisation, antibacterial and bioactivity properties of experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes

Article Type: Full Length Article

Keywords: Adhesives; Antibacterial; Bioactivity; Nanotubes; Orthodontics; Triclosan.

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First Author: Felipe Weidenbach Degrazia

Order of Authors: Felipe Weidenbach Degrazia ; Bruna Genari; Vicente Castelo Branco Leitune ; Rodrigo A Arthur; Santiago Arias Luxan ; Susana M Werner Samuel ; Fabrício Mezzomo Collares; Salvatore Sauro, Ph.D

Abstract: Objective. To evaluate the immediate enamel bond strength, in situ degree of conversion and the polymerisation rate of three experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes. The antibacterial and bioactivity properties of such experimental materials were also assessed.
Materials and Methods. Three experimental orthodontic adhesives were formulated by incorporating triclosan-loaded halloysite nanotubes (TCN-HNT) at different concentrations (5 wt%, 10 wt% and 20 wt%) into resin blend (Control). The maximum polymerisation rate of the tested adhesives was evaluated trough FTIR, while Raman was used to analyse the in situ degree of conversion (DC) at the bracket/enamel interface. The shear bond strength (SBS) of the enamel-bonded specimens was assessed at 24 h. The antibacterial properties of the experimental materials against S. Mutans were evaluate up to 72h, while, their bioactivity was evaluated after 14 days of artificial saliva (AS) storage through SEM-EDS and Raman spectromicroscopy.
Results. Incorporation of TCN-HNT increased the polymerisation properties without interfering with the immediate bonding properties of the experimental adhesives. All experimental adhesives containing TCN-HNT inhibited bacterial growth at 24h, and induced mineral deposition after 14 days of AS storage. At 72 h, only the experimental system containing 20% TCN-HNT maintained such a capability.
Conclusions. Adhesives doped with TCN-HNT present improved polymerisation properties and suitable bonding performance. However, adhesives containing TCN-HNT >10% might promote long-term antibacterial activity and reliable mineral deposition.
Significances: The use of adhesives containing triclosan-loaded halloysite represent a promising "smart" approach to bond orthodontic brackets and bands; these might prevent enamel demineralisation and induce enamel remineralisation during the treatment.

July 25th, 2017

Prof. Dr. Cristopher D. Lynch

Editor-in-Chief Journal of Dentistry

Dear Prof. Dr. Cristopher D. Lynch,

On behalf of my co-workers Felipe Weidenbach Degrazia, Bruna Genari, Vicente Castelo Branco Leitune, Rodrigo Alex Arthur, Santiago Arias Luxan, Susana Maria Werner Samuel, Fabrício Mezzomo Collares, I would like to submit this manuscript titled "*Polymerisation, antibacterial and bioactivity properties of experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes*" to **Journal of Dentistry**.

This manuscript is original and it has not been published in any form or language, but it is only submitted for publication to the **Journal of Dentistry**. The authors are completely free of conflicts of interest.

Our groups of research collaborated to evaluate the enamel bond strength, in situ degree of conversion and the polymerization rate of several experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes. The antibacterial and bioactivity properties of such experimental materials were also assessed. This is the first study where an innovative experimental orthodontic adhesive containing triclosan-loaded halloysite nanotubes (TCN-HNT) was generate and assess.

The results of this study showed that the experimental adhesives containing triclosan-loaded halloysite nanotubes inhibited bacterial growth at 72h and mineral deposition (Bioactivity). Moreover, the incorporation of 20 wt.% TCN-HNT increases the degree of conversion and the shear bond strength to enamel compared to a commercial group Transbond XT. Thus, the incorporation of TCN-HNT may promote long-term antibacterial activity and mineral deposition, and reliable physicochemical

properties of orthodontic adhesives. Such innovative orthodontic adhesives may be a promising material for preventing demineralization and increasing the therapeutic success of orthodontic treatments.

Thank you very much for your kind attention

Yours sincerely,

Salvatore Sauro

Prof. Dr. Salvatore Sauro - Corresponding author

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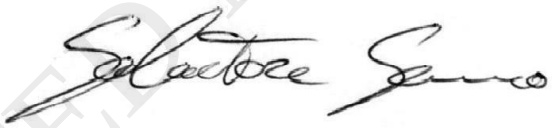
- Hon. Senior Lecturer - Biomaterials, Biophotonics and Tissue Engineering, King's College London Dental Institute (KCLDI). Floor 17 Tower Wing, Guy's Hospital, Great Maze Pond. London SE1 9RT (UK)



October 30th, 2017

Ref.: Ms. No. JJOD-D-17-00749
Polymerisation, antibacterial and bioactivity properties of experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes

Professor. Christopher D. Lynch
Editor in Chief
Journal of Dentistry

Firstly, the authors would like to thank the time spent reviewing our manuscript. The answers for the comments of the reviewers are below point-by-point in *red*. The manuscript was also marked with words underlined.

In this regard, we would like to show all our gratefulness for your constructive comments that were fair and helpful.

Reviewer #1: This is an interesting paper that evaluated the immediate enamel bond strength, in situ degree of conversion and the polymerisation rate of three experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes. The antibacterial and bioactivity properties of such experimental materials were also assessed. The research design is sound and the results are clearly presented. Here are some comments for you to consider.

- we would like to show all our gratitude for your constructive comments that were fair and helpful.

Materials and methods

How was the concentration of TCN-HNT filler selected?

- *A: We made a screening of concentrations of Halloysite based on a previous study published by Degrazia et al, 2016 where the authors used Triclosan into Halloysite nanotubes . A study of Bottino et al. (2013) showed that the percentage of Halloysite nanotubes above 20 wt% incorporated into a dental adhesive decrease its properties.*

References: Bottino MC, Batarseh G, Palasuk J, Alkatheeri MS, Windsor LJ, Platt JA.. Nanotube-modified dentin adhesive-physicochemical and dentin bonding characterizations. Dent Mater 2013;29:1158-1165.

- *Degrazia FW, Leitune VCB, Takimi AS, Collares FM, Sauro S. Physicochemical and bioactive properties of innovative resin-based materials containing functional halloysite-nanotubes fillers. Dent. Mater. 2016;32:1133-1143.*

Pls add the manufacturer information for 37% Phosphoric acid.

- *A: The authors added the manufacturer information for 37% phosphoric acid: Acid Gel, Villevie, Joinville, SC, Brazil.*

Pls specify the brand name of orthodontic brackets used.

- *A: The authors added the brand name of orthodontic brackets used: (Morelli Ltd, Sorocaba, SP, Brazil).*

Were the data assessed for normality and equality of variance?

- *A: All data were assessed for normality and equality of variance using Shapiro-Wilk and Brown-Forsythe tests, respectively. The authors are sending with the response the output in .pdf of tests (see supplementary material uploaded in the J dent website). The information of normality and equality of variance was added in the "Statistical analysis" sub-section: "The normality of data was evaluated using Shapiro-Wilk test ($p > 0.05$ for all tests). Homogeneity of variance was calculated using the Brown-Forsythe test. For all tests the variances were homoscedastic ($p > 0.05$)."*

Pls specify the factors assessed using two-way ANOVA.

- *A: The factors assessed by two-way ANOVA are Concentration of TCN-HNT x Time (h) of the colony forming units CFU (dependent variable). The authors specified this information in the text: "Two-way ANOVA and Tukey's post hoc were used to assess differences in the microbiology assay (log_{10} CFU/mL) in the concentrations of TCN-HNT and different incubation periods."*

Results

2nd paragraph Line 1 Pls replace "table 1" with "Table 1".

- *A: The authors replaced "table 1" with "Table 1".*

2nd paragraph Line 2 Pls replace "74.05 to 76.85" with"74.05 to 76.72".

- *A: The DC of "74.05 to 76.85" found between TCN-HNT groups was changed to "74.05 to 76.72".*

What are the results of 2-Way ANOVA? Are the two factors and their interaction significant?

- *A: Thank you for the observation. We added the results of two-way ANOVA into the Table 1 and in the text. Both factors and their interaction were significant with $p < 0.05$. We have also added the information of factors and their interaction to the "Results" and "Discussion" sections, respectively: "During the period of incubation a decreased antibacterial activity was found after 48 and 72 h for all experimental groups compared to*

24 h ($p < 0.05$).” “Although a decreased of antibacterial activity after 72 h compared to 24 h of incubation for the experimental groups, the prolonged diffusion of triclosan from the nanotubes may have diffused over time into bacterial membrane, improving the antimicrobial capability of the experimental adhesive containing 20% of TCN-HNT [37].”

- *A file of the statistical analysys has been uploaded as supplementary material*

How much triclosan is released from the TCN-HNT fillers? What is the relationship between the concentration of TCN-HNT fillers and the amount of triclosan released?

- *A: To have effective benefit of the gradual and slow release properties of loaded agents into Halloysite nanotubes the amount of Triclosan mixed with agents (in this case Halloysite nanotubes) in a 1:1 ratio (in weight) was based on previous studies (Bottino et al., 2013; Degrazia et al., 2016). One reason of the 1:1 ratio was following manufacturer specifications (Sigma Aldrich) where we find data of the molecular weight of Halloysite (294.2g/mol) is similar to triclosan (289.5g/mol). A previous study (Bedran et al., 2014) has demonstrated the minimum inhibitory concentration (MIC) of triclosan (7.8 µg/ml) for S. Mutans and that the biofilm formation of S. Mutans is dose-dependent induced by sub-minimum inhibitory concentrations of triclosan at 1/2 and 1/4. Higher concentrations of TCN-HNT added to adhesive (above MIC) promoted increased and longer antimicrobial effects. Since Halloysite nanotubes have no antibacterial properties, the antibacterial effect was promoted only due to release of triclosan from TCN-HNT.*

References: - Bottino MC, Batarseh G, Palasuk J, Alkatheeri MS, Windsor LJ, Platt JA. Nanotube-modified dentin adhesive-physicochemical and dentin bonding characterizations. Dent Mater 2013;29:1158-1165.
- Degrazia FW, Leitune VCB, Takimi AS, Collares FM, Sauro S. Physicochemical and bioactive properties of innovative resin-based materials containing functional halloysite-nanotubes fillers. Dent. Mater. 2016;32:1133-1143.
- Bedran TB, Grignon L, Spolidorio DP, Grenier D. Subinhibitory concentrations of triclosan promote Streptococcus mutans biofilm formation and adherence to oral epithelial cells. PLoS One. 2014:13;9:e89059.

Discussion

Why would the incorporation of TCN-HNT fillers into the experimental orthodontic adhesives induced a significant increase of the degree of conversion?

- *A: One explanation could be that during the analysis by Raman spectroscopy the TCN-HNT fillers would have warmed-up and consequently promoted a photocatalytic effect. Another reason is that increased kinetics may be associated with the increase of viscosity of the reacting system. As filler content increases and viscosity rises, radical mobility is reduced which significantly decreases the probability of bi-radical termination and therefore autoacceleration occurs more quickly and at a higher rate (Hadis et al., 2011; Leprince et al., 2010; Leprince et al., 2011). Furthermore, a previous report (Peng et al.., 2017) has demonstrated the development of a photo-catalyst system of a composite with Halloysites*

in which light mediation synergistically regulate the carrier generation and charge transfer to enhance photoactivity.

References: - Hadis M, Leprince JG, Shortall AC, Devaux J, Leloup G, Palin WM. High irradiance curing and anomalies of exposure reciprocity law in resin-based materials. Journal of Dentistry 2011;39:549–57.
- Leprince JG, Lamblin G, Devaux J, Dewaele M, Mestdagh M, Palin WM, et al. Irradiation modes' impact on radical entrapment in photoactive resins. Journal of Dental Research 2010;89:1494–8.
- Leprince JG, Hadis M, Shortall AC, Ferracane JL, Devaux J, Leloup G, et al. Photoinitiator type and applicability of exposure reciprocity law in filled and unfilled photoactive resins. Dental Materials 2011;27:157–64.

- Peng H, Liu X, Tang W, Ma R. Facile synthesis and characterization of ZnO nanoparticles grown on halloysite nanotubes for enhanced photocatalytic properties. Scientific Reports 2017;7:2250.

The antibacterial properties of the experimental adhesives are only tested for 72 h, what will the long-term antibacterial effect be like?

- *A: The period of evaluation of major in vitro studies lasts 24 h of contact between specimen and bacteria. Firstly, we have prolonged this period up to 96 h of study. However, after 96 h a great portion of bacteria died and no CFU was possible to count. Otherwise, at 72 h sufficient CFU count was possible. This in vitro study of experimental adhesive specimens in contact with supersaturated medium of S. mutans during 72 h would be challenging to last for great periods in the mouth. Besides, the release of antibacterial agents loaded into nanotubes is gradual and slow increasing their period of efficacy (Kamble et al., 2012; Liu et al., 2014). The clinical long-term effect of antibacterial adhesives should promote prevention of white spot lesion around orthodontic brackets. Moreover, the duration of antibacterial effects of long-term clinical studies is not known and no clinical studies are available to determine whether the duration of antibacterial effects of orthodontic adhesives observed in vitro suffice to increase the prevention of white spot lesions. The next steps of our studies are to designs in situ and in vivo studies to answer to this issues*

References: - Priyadharshini S, Ahmed A, Savadamoorthi S. In vitro antibacterial effectiveness of three different dentin bonding systems against Streptococcus mutans and Enterococcus faecalis. Journal of International Oral Health 2017;9:33-37.
- Kamble R, Ghag M, Gaikawad S, Panda BK. Halloysite Nanotubes and Applications: A Review. Journal of Advanced Scientific Research 2012;3: 25-29.
- Liu M, Jiab Z, Jiab D, Zhoua C. Recent advance in research on halloysite nanotubes-polymer nanocomposite. Progress in Polymer Science 2014; 39:1498–1525.

What are the limitations of the present study?

A: One limitation of this study is the antibacterial effect that could not be evaluated for more than 72 h due to the insufficient number of bacteria available in the medium.

The authors inserted this limitation in the "Discussion" section:

"The limitation of this study was the antibacterial effect that could not be evaluated due to bacteria death after 72 h."

Figure 1: Legends for Fig 1B missing

- *A: Legends for Fig 1B was inserted.*

Figure3: Figure 3e missing

- *A: Figure 3e is placed under Figure 3a. To better understanding we changed the legend for Figure 3e.*

"After saliva immersion, no mineral deposition was observed in control group (image e)"

Figures 3 and 4: images needed improvement, cannot be clearly seen.

- *The authors agree with the reviewer and improved the resolution of images from Figures 3 and 4, rephrased legends and clarify indications in the figures.*

Reviewer #2: This study evaluated the immediate enamel bond strength, in situ degree of conversion and the polymerization rate, the antibacterial and bioactivity properties of triclosan-loaded halloysite nanotubes as orthodontic adhesives. In the perspective of immediate bonding properties, TCN-HNT with different concentration did not show significant difference compared with TCN-free adhesive. Moreover, the addition of TCN increased the polymerization property and antibacterial property. Besides, TCN-HNT could induce the deposition of minerals on etched enamel.

However, the evidences were too weak to support the clinical significance. Here is some advices for your consideration:

1.The HNTs has been incorporated into dental adhesives as nano-carries for antibacterial agent before. So, this study is lack of novelty.

- *A: Thank you or your concern, but unfortunately we have to disagree with this observation. In Dentistry field only two papers added Halloysite nanotubes with antimicrobial agents into adhesives (Feitosa et al., 2014; Degrazia et al., 2016). However, one of them (Feitosa et al., 2014) evaluated the use of Doxycicline in a dentin adhesive and the second one (Degrazia et al., 2016) has not evaluated the antibacterial effect of the adhesive. To avoid formation of white spot lesions during orthodontic treatment the necessity of testing adhesives with properties of antimicrobial loaded agents is fundamental and novel in the Orthodontic field. Halloysite nanotubes have been used as nanocarriers for antibacterial agents due to its slow and gradual release.*

References: - Feitosa SA, J. Palasuk, K. Kamocki, S. Geraldeli, R.L. Gregory, J.A. Platt et al. Doxycycline-Encapsulated Nanotube-Modified Dentin Adhesives. J Dent Res. 2014;93:1270–1276.
- Degrazia FW, Leitune VCB, Takimi AS, Collares FM, Sauro S. Physicochemical and bioactive properties of innovative resin-based materials containing functional halloysite-nanotubes fillers. Dent. Mater. 2016;32:1133-1143.

2.The resolution of the four figures provided in the article is too low. I could not get useful information as you described in the Results section. From the SEM figures, we can just say there is random mineral deposition, but not remineralization. So, there maybe no use for preventing enamel demineralization. Besides, I suggest you to rewrite you figure legends so that we could understand the information you want to show us.

A: The authors agree with the reviewer and reconstructed the images with a resolution of 600 dpi.

- *The authors also changed term "remineralisation" to "mineral deposition" and rephrased the figures caption: "Figure 1. (a) TEM image of a nanotube with its inner-surface of 40–50 nm diameter range and outer-surface length of 90 nm diameter. (b) TEM image show the presence of TCN nanoparticles inside nanotubes. It is also possible to observe empty dark spaces, which may permit the mobility of triclosan through the tube."*
- *"Figure 3. SEM images with EDS scales of mineral deposition. In images a, b, c and d, no mineral deposition was observed prior artificial saliva. e) No mineral deposition after saliva immersion in control group. In images f, g and h, mineral deposition was observed in adhesives containing respectively 5%, 10% and 20% of TCN-HNT after 14 days of saliva immersion signalized by an orange target and confirmed by EDS scales. Dark line highlights interface between adhesive (A) and enamel (E) areas."*
- *"Figure 4. Analyses of interface between adhesive and enamel surface with different TCN-HNT concentrations, initially and after 14 days. Arrows indicate mineral deposition on interface with adhesive containing 5%, 10% and 20% of TCN-HNT after 14 days of immersion in artificial saliva. Peaks of 960 cm^{-1} and 1610 cm^{-1} represent calcium and methacrylate chemical groups, respectively. Crossing lines represent interface, E represents enamel side and A represents adhesive side."*

3.Figure 2 told me that the adhesive remaining of 20%wt HNT/TCN group was significantly different from the other three groups. However, in the Results section and Discussion section, you said the adhesive failure of 20%wt HNT/TCN group was greater. I was a little confused that what is the connection between adhesive remaining and adhesive failure?

- *A: The adhesive remaining on the enamel surface of 20%wt HNT/TCN group decreased compared to other groups. Since no adhesive was found in almost 60% of samples of the 20%wt HNT/TCN group a higher number of adhesive failures (E-score 1 and/or 4) were found compared to higher values of cohesive failures percentages of the other groups (E-score 2 and/or 3).*

We rephrased this sentence to clarify understanding: "It was observed a significant decrease of adhesive remaining on enamel ($P < 0.05$) in specimens bonded with the adhesive containing 20 % TCN-HNT compared to all the other groups."

4.Taken all your results into consideration, I thought the advantages of your HNT/TCN adhesive system were not prominent. The addition of TCN could promote the disposition of minerals as you have shown. However, whether TCN could improve the remineralization of etched enamel and whether there was difference between normal enamel and remineralized enamel were unclear. I suggest you to demonstrate the clinical significance of this adhesive system.

- *A: The attraction of mineral deposition may inhibit enamel demineralization since these depositions would serve as source of ions to keep the environment pH instead of Ca^{+} and P^{-} ions of dental enamel. We believe that these materials could postpone enamel demineralization. The authors added a sentence in the "Discussion" section to clarify the clinical significance of this adhesive system: "Brackets and archwires usually create numerous retention sites hampering tooth cleaning and increasing potential to develop white spot lesions on enamel around them [31,32]."*

References: 31. E. Tufekci, J.S. Dixon, J.C. Gunsolley, S.J. Lindauer, Prevalence of white spot lesions during orthodontic treatment with fixed appliances. Angle Orthod. 81 (2011) 206-210.

5. There were some grammatical mistakes in your article. Please check them out. Such as "Thus, the first objective of this this study was"…

- *A: The authors agree with the reviewer. The text was checked and grammatical mistakes were corrected.*

In summary I'd like to suggest you to refine your study design and add more evidence in order to provide some reference to clinicians.

- *A: The authors agreed with the reviewer and added more clinical evidence based on the article published by E. Tufekci, J.S. Dixon, J.C. Gunsolley, S.J. Lindauer, Prevalence of white spot lesions during orthodontic treatment with fixed appliances. Angle Orthod. 81 (2011) 206-210.*

Thank you for such a careful review of our manuscript.

We hope these changes have made the manuscript acceptable for publication. We thank the reviewers for helping us to improve the paper.

Best Regards

Polymerization, antibacterial and bioactivity properties of an experimental orthodontic adhesive containing triclosan-loaded halloysite nanotubes

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SHORT TITLE*:* Triclosan-loaded halloysite nanotubes, a filler for innovative orthodontic adhesives

Keywords: Adhesives; Antibacterial; Bioactivity; Nanotubes; Orthodontics; Triclosan.

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Polymerisation, antibacterial and bioactivity properties of experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes

Role of the eight Authors of this paper

1st Felipe Weidenbach Degrazia: ROLE: contributed to conception, design, data acquisition, analysis, and data interpretation, drafted, gave final approval and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

2nd Bruna Genari: ROLE: contributed to conception, data acquisition, critically revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

3rd Vicente Castelo Branco Leitune: ROLE: contributed to conception, design, data interpretation, critically revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

4th Rodrigo Alex Arthur: ROLE: specimen preparation and data acquisition, revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

5th Santiago Arias Luxan: ROLE: contributed data interpretation, statistical analysis, revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

6th Susana Maria Werner Samuel: ROLE: Specimen preparation and data acquisition, revised the manuscript and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

7th Fabrício Mezzomo Collares: ROLE: contributed to conception, design, and data interpretation, drafted, critically revised the manuscript, gave final approval and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

8th Salvatore Sauro: ROLE: contributed to conception, design, data acquisition, analysis, and data interpretation, drafted, critically revised the manuscript, gave final approval and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Polymerisation, antibacterial and bioactivity properties of experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes

ABSTRACT

Objective. To evaluate the immediate enamel bond strength, *in situ* degree of conversion and the polymerisation rate of three experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes. The antibacterial and bioactivity properties of such experimental materials were also assessed.

Materials and Methods. Three experimental orthodontic adhesives were formulated by incorporating triclosan-loaded halloysite nanotubes (TCN-HNT) at different concentrations (5 wt%, 10 wt% and 20 wt%) into resin blend (Control). The maximum polymerisation rate of the tested adhesives was evaluated trough FTIR, while Raman was used to analyse the *in situ* degree of conversion (DC) at the bracket/enamel interface. The shear bond strength (SBS) of the enamel-bonded specimens was assessed at 24 h. The antibacterial properties of the experimental materials against *S. Mutans* were evaluate up to 72h, while, their bioactivity was evaluated after 14 days of artificial saliva (AS) storage through SEM-EDS and Raman spectromicroscopy.

Results. Incorporation of TCN-HNT increased the polymerisation properties without interfering with the immediate bonding properties of the experimental adhesives. All experimental adhesives containing TCN-HNT inhibited bacterial growth at 24h, and induced mineral deposition after 14 days of AS storage. At 72 h, only the experimental system containing 20% TCN-HNT maintained such a capability.

Conclusions. Adhesives doped with TCN-HNT present improved polymerisation properties and suitable bonding performance. However, adhesives containing TCN-HNT >10% might promote long-term antibacterial activity and reliable mineral deposition.

Significances: The use of adhesives containing triclosan-loaded halloysite represent a promising "smart" approach to bond orthodontic brackets and bands; these might prevent enamel demineralisation and induce enamel remineralisation during the treatment.

1. INTRODUCTION

Enamel demineralisation is one of the main causes responsible for the formation of white spot lesions (WSL) during fixed orthodontic treatments, in particular in those patients with limited oral hygiene compliance [1]. Wide gaps are often observed at the adhesive–enamel junction around brackets [2]; this represents the most common site for demineralisation to occur due to accumulation of a biofilm riches in cariogenic species such as *Streptococcus mutans* [3,4].

It has been advocated that the use of ion-releasing materials such as glass ionomer cements may reduce the risk of enamel demineralisation and prevent excessive bacterial growth [5]. Based on this information, several therapeutic agents such as fluoride [6], chlorhexidine [7] and nano-silver [8] have been incorporated into experimental orthodontic bonding materials to overcome such a clinical issue. However, it seems that although their early effectiveness, the release of such active principles tends to reduce overtime. Moreover, some of these agents may induce tooth discoloration; for instance, the release of silver ions or chlorhexidine may often cause anaesthetics appearance when incorporated into orthodontic adhesives [9].

It has been reported that the use of triclosan nanoparticles may represent an alternative to conventional antibacterial agents due to its small particle size as well as to the little amount needed to produce antibacterial effects [10]. Several researchers have been inspired to create innovative composites containing such inorganic nanoparticles due to their large surface area and to their high surface reactivity [11,12].

Furthermore, nano-compounds based on mesoporous aluminosilicate clay (i.e. halloysite nanotubes - HNTs) have been incorporated into dental adhesives as nano-carries for antibacterial agents [13]. Moreover, these nanotubes have an inner diameter of 30-70 nm, which could be infiltrated by resin monomers and increase the mechanical properties of resin-based materials due to their great elastic modulus (140 GPa) [14].

We hypothesised that triclosan-loaded HNTs might be used as a promising nano-filler for innovative therapeutic orthodontic adhesives to induce mineral deposition on the enamel around brackets.

Thus, the first objective of this study was to evaluate the enamel bond strength, and the polymerisation properties (i.e. *in situ* degree of conversion and polymerisation rate) of experimental orthodontic adhesives containing different concentrations of triclosan-loaded halloysite nanotubes. The antibacterial and bioactivity properties of such experimental materials doped with triclosan-loaded HNTs were also assessed.

The first hypothesis tested in this study was that the incorporation of triclosan-loaded HNTs within the composition experimental orthodontics adhesives would not interfere with their immediate bonding performance to acid-etched enamel and the polymerisation properties compared to the control experimental adhesive containing no TCN-HNT.

The second hypothesis was that the incorporation of different amount of triclosan-loaded HNTs would enhance the antibacterial, and bioactivity properties of such materials compared to the control experimental adhesive containing no TCN-HNT.

2. MATERIALS AND METHODS

2.1. Preparation of TCN-loaded HNTs (TCN-HNT)

Halloysite nanotube (HNT) – $Al_2Si_2O_5(OH)_4.2H_2O$ with a diameter of 30-70 nm and length of 1-3 μm (Sigma-Aldrich, St. Louis, MO, USA) were submitted to a silanisation process using a solution containing 5 wt.% of 3-metacryloxypropyltrimetoxysilane and 95 wt.% acetone at 110 °C for 24 h. Subsequently, the treated nanoparticle were mixed [1:1 ratio] with 2,4,4-Trichloro-2-hydroxydiphenyl ether (TCN – Triclosan, Fagron, Rotterdam, SH, Netherlands) under continues agitation for 1 h as described in a previous study [15]. The mixture was then dispersed in 95 wt.% pure ethanol (0.03 mg ml^{-1}) at 30 °C and sonicated for 1 h. Subsequently, the TCN-HNT nanotubes were desiccated for 10 days at 30 °C to ensure complete evaporation of residual solvents [15]. TCN-HNT was finally characterised using a Transmission Electron Microscope (TEM) JEM 120 Exll (JEOL, Tokyo, Japan) at 80 kV at a magnification X 300,000.

2.2. Formulation of experimental adhesive - incorporation of TCN-HNT

A monomer resin blend was created by mixing 75 wt.% bisphenol-A-glycol dimethacrylate (BisGMA - Sigma-Aldrich Co., St. Louis, MO, USA) and 25 wt.% triethylene glycol dimethacrylate (TEGDMA- Sigma-Aldrich) and used as a control adhesive containing no TCN-HNT. Moreover, a photoinitiator (CQ: Camphorquinone, 0.5 mol%) and two co-initiators (EDAB: ethyl 4-dimethylaminobenzoate; DPIHFP: diphenyliodonium hexafluorophosphate)

(Aldrich Chemical Co., Milwaukee, WI, USA) were also added at a concentration of 1 mol%.

The TCN-HNT filler was added at concentrations of 5, 10 and 20 (wt.%) into the resin blend in order to create three experimental adhesives. All formulations were mixed and maintained in an ultrasonic bath for 1 h. The four experimental resin adhesives were finally stored in a dark chamber.

2.3. Polymerisation rate evaluation (FTIR-ART)

Polymerisation rate of the experimental adhesives was evaluated using Fourier transform infrared spectroscopy (FTIR) using a spectrometer equipped with an attenuated total reflectance (ATR) device (Vertex 70; Bruker Optics, Ettlingen, Germany). Three specimens for each group were analysed by directly applying the tested materials (3 μL) onto the diamond crystal. These were light activated for 40 s using a light emitting diode unit (Radii Cal, SDI, Bayswater, VIC, Australia) with an irradiation value of 1200 mW/cm^2 at a standardised distance of 2 mm. Analysis was performed at a controlled room temperature of 23±1°C and 60±1% relative humidity.

The IR-Solution software was used to standardise the assessment parameters such as scanning range (4000–800 cm^{-1}), resolution (4 cm^{-1}), and scanning time (40 s). This setup allowed the acquisition of each single scan, every 0.7 s during photo-activation procedure. The maximum polymerization rate [Rp.(s^{-1})] was also evaluated as described in a previous study [16] based on the intensity of the C=C stretching vibrations at 1635 cm^{-1} (peak height) as a function of time, and using the symmetric ring stretching at 1608 cm^{-1} from the polymerised and non-polymerised samples as an internal standard.

The data was plotted and a sigmoidal curve fitting method was applied with the linear regression method using Sigma Plot 12.0 for Windows (Systat Software Inc, San Jose, CA, USA).

2.4. Bonding procedures and bond strength evaluation

The buccal surface of 60 anterior bovine teeth were polished using a 600 grid SiC paper for 5 s in order to remove the aprismatic enamel only; this was checked by using a stereo microscope (X80). All specimens were then individually placed in cylinder-shape moulds and embedded in a self-curing polymethyl-methacrylate resin (Esschem Linwood, Pennsylvania, PA, USA), with buccal surface perpendicular to the horizontal plane; fifteen enamel specimens were used for each adhesive group (Table 1). The enamel surface of each specimen was first etched for 30 s with a 37% phosphoric acid gel (Acid Gel, Villevie, Joinville, SC, Brazil) and rinsed with water for the same time. After air-drying for 5 s, orthodontic brackets were positioned on the enamel surface and kept under constant pressure (276 g); resin excess around the bracket was removed with a sharp dental probe.

A light-curing procedure was performed for 40 s [17] using a light emitting diode unit (Radii Cal, SDI) with an irradiation value of 1200 mW/cm^2.

Specimens bonded to their brackets (Morelli Ltd, Sorocaba, SP, Brazil) with 11.18 mm^2 area, were stored in distilled water at 37 °C for 24 h. Subsequently, these were positioned in a Universal Testing Machine (Shimadzu EZ-SX, São Paulo, SP, Brazil) positioning the incisal edge of the bracket base parallel to a sharpened chisel blade. They were stressed to failure using a 500 N load cell with a crosshead speed of 1.0 mm/min. The maximum force required for bracket's

debonding was recorded in N, which was used to calculate the bond strength in MPa (N/mm^2). Following debonding, the amount of adhesive on each enamel surface was with analysed using a stereomicroscope (X10) and the adhesive remnant index (ARI) calculated as described in a previous study [18].

2.6. In situ Degree of conversion - micro-Raman assessment (DC)

Three teeth for each group were prepared and bonded as previously describe. These were subsequently sectioned in two halves after 24 h of storage in distilled water at 37°C. The specimens were then submitted to the *in situ* analysis of the DC along the bracket-enamel interface. This analysis was accomplished using micro-Raman spectroscopy using the Senterra equipment (Bruker Optik GmbH, Ettlingen, Germany) for five times (3 s) by a 100-mW diode laser with 785-nm wavelength. Firstly, it was collected the spectra of each un-polymerised adhesive at three different individual points, and subsequently, the spectra of the polymerised adhesive was obtained at three different sites along the bracket-enamel interface. The aliphatic and aromatic peaks at 1635 cm^{-1} and 1608 cm^{-1} were considered, respectively. The average value of the measurements from the same group was used to calculate the ratio of double bond content after polymerisation.

2.7. Mineral deposition – Bioactivity assay

The same specimens prepared for the *in situ* DC assay were analysed again through micro-Raman spectroscopy using the same parameters as aforementioned. The specimens were then immersed for 14 days in artificial saliva (AS) [19]. The specimens were analysed through eight scanning lines (20

μm distance) running perpendicularly along the adhesive-enamel interface for an area of 100 μm x 160 μm. The aromatic peak at 1608 cm^{-1} and the phosphate peak at 962 cm^{-1} were integrated and the mean value per group was plotted in graphs to evaluate mineral deposition at the bracket-enamel interface. Scanning Electron Microscopy (SEM) with Energy Dispersive Spectroscopy (EDS) was also performed to characterise the content of the minerals deposited along the interface after 14 days of AS storage.

2.8. Microbiology assay

Thirty-six specimens (4 mm x 1 mm) from each experimental group (n=9) were fixed on 6 x 6 x 8 mm teflon matrices and attached to the lid of a 48-well plate. This device was similarly used by previous studies [20, 21] to allow bacteria grown on the surface of tested samples. The plate was sterilised using ethylene oxide. Each well was filled with 500 μL of brain-heart infusion (BHI) broth supplemented with 0.5% of sucrose and 50 μL of inoculum, which was prepared adjusting *Streptococcus mutans* (UA159) to the optical absorbance of 0.3. The cover was placed with specimens in contact to the inoculum, immersed in BHI broth and incubated at 37 ºC for 24h.

Afterward, three disks for each group were transferred to a microtube containing 900 μL of sterile saline solution (0.9% NaCl) and the biofilms were harvested. The bacterial suspensions were serially diluted (100 μL) in sterile saline solution. Two aliquots of 25 μL were placed onto a BHI agar, incubated anaerobically at 37 ºC for 48 h, followed by evaluation of the biofilm colony-forming unit (CFU). The other discs were incubated into a new 48-well plate with

fresh medium and incubated at 37 °C for 24 h, completing 48 h of incubation, and sequentially up to 72 h (n=3). At these periods, the described above protocol of serial dilution and plate onto agar was repeated.

2.9. Statistical analysis

The normality of data was evaluated using Shapiro-Wilk test ($p > 0.05$ for all tests). Homogeneity of variance was calculated using the Brown-Forsythe test. For all tests the variances were homoscedastic ($p > 0.05$). Two-way ANOVA and Tukey's post hoc were used to assess differences in the microbiology assay (log_{10} CFU/mL) in the concentrations of TCN-HNT and different incubation periods. One-way ANOVA and Tukeys' post-hoc were used for the statistical analysis of the data obtained during the DC, maximum polymerisation rate and shear bond strength tests. ARI score values were evaluated using the Kruskal-Wallis and Tukey's post hoc tests.

3. RESULTS

The TEM analysis showed the overall morphology and confirmed the nano-dimension of halloysite nanotubes (Figure 1A) as well as the successful functionalisation of Triclosan inside lumen of the halloysite nanotubes (Figure 1B).

The results of *in situ* DC are depicted in Table 1 and these varied from 74.05 to 76.72. It was showed that there was no significant difference in DC between the experimental groups ($P > 0.05$) of TCN-HNT compared to the control resin blend containing no TCN-HNT nanotubes. The $R_p.(s^{-1})$ reduced

significantly with TCN-HNT concentration >10% compared to the control resin blend containing 0% and the experimental 5% TCN-HNT (Table 1).

Shear bond strength mean and standard deviation are expressed in MPa and presented in table 1. The adhesive containing no TCN-HNT and that with 5% TCN-HNT showed the highest results, although no significant difference was observed between the groups tested in this study ($P > 0.05$). The ARI score is presented in Figure 2. It was observed a significant decrease of adhesive remaining on enamel ($P < 0.05$) in specimens bonded with the adhesive containing 20 % TCN-HNT compared to all the other groups. While, no significant difference ($P > 0.05$) was found between the experimental resins containing 0, 5 and 10 % TCN-HNT.

SEM-EDS analysis showed mineral deposition in the adhesives containing 5%, 10% and 20% of TCN-HNT after 14 days of saliva immersion (Figure 3). However, the greatest mineral precipitation at the adhesive-enamel interface was observed when using the experimental resins containing 10% and 20% TCN-HNT (Figure 4).

The results of the microbiology analysis (log_{10}CFU/mL) are shown in Table 1. At 24h, adhesives with 5%, 10% and 20% of TCN-HNT differed from the resin containing 0% TCN-HNT ($P < 0.05$). It was found that the greater the concentration of TCN-HNT within the adhesive composition the lower CFU counts. Indeed, adhesives with 10% and 20% of TCN-HNT presented lower CFU counts ($P < 0.05$) at 48 h compared to all the other groups. After 72 h of incubation, only the adhesive with 20% of TCN-HNT maintained its antimicrobial activity. During the period of incubation a decreased of antibacterial activity was found after 48 and 72 h for all experimental groups compared to 24 h ($p < 0.05$).

4. DISCUSSION

It is well-know that the prevalence of white spot lesions formation in enamel may increase during orthodontic treatment [22]. This is the reason why we believe that the use of antibacterial/remineralising bonding materials may prevent, or at least reduce, enamel demineralisation during fixed orthodontic treatments.

It has been demonstrated that halloysite nanotubes can be used as functional fillers for resin-based materials due to several advantages. These include relatively low production cost, natural availability and their suitability as drug carrier [11].

This study showed that triclosan-loaded halloysite nanotubes could be successfully incorporated within the composition of experimental resin-based adhesives. The bond strength results obtained in this study showed no substantial alteration of the immediate bonding performance after incorporation of different amounts of TCN-HNT into the experimental adhesives compared to the experimental adhesive containing no TCN-HNT. Moreover, TCN-HNT incorporation induced an increase of the degree of conversion (DC) of the experimental adhesives compared to the one containing no TCN-HNT. However, similar degree of conversion (DC) could be observed among all the experimental orthodontic adhesives containing the TCN-HNT at different concentration. Conversely, the highest polymerisation rate between all the experimental orthodontic adhesives containing TCN-HNT was observed with the system containing 5 wt% of TCN-HNT. The lower polymerisation rate observed in the experimental adhesives containing TCN-HNT >5wt% compared to the control resin blend may have been caused by some sort of interference with the

scattering of the light during light-curing procedures; it is well-known that the higher the filler amount the lower the diffusion of the light thought the resin bulk [13, 14]. However, high cross-linking density leads to increase mechanical stability, lowering the degradation of polymeric matrix [23, 24].

Such a situation was also evident during the bond strength test. Indeed, the incorporation of TCN-HNT >5 wt% caused a reduction of the bonding performance of the tested experimental adhesive. Although such a reduction was not significantly, we hypothesised that it was caused by an excessive presence of nanoparticles that may have deposited within the porosities of the acid-etched dentine and interfered with the diffusion of the resin monomer and with a proper hybrid layer formation. Indeed, in the specimens bonded with the adhesive containing 20 wt% TCN-HNT, it was observed a higher failure in adhesive mode compared to other groups in this study (Figure 2). However, our results are in accordance with the results of a recent meta-analysis [25], where it is affirmed that the incorporation of antimicrobial agents into an orthodontic adhesive does not influence bond strength to enamel. It was also demonstrated that higher SBS may lead to sound enamel loss during bracket debonding [26]. However, the bond strength results obtained with 10 and 20 wt% of TCN-HNT were higher than those considered clinically acceptable [27,28]; this may also reduce the risk for critical enamel loss during bracket debonding [29].

Therefore, the hypothesis that the incorporation of triclosan-loaded HNTs within the composition experimental orthodontics adhesives would not interfere with their immediate bonding performance and the polymerisation properties of experimental orthodontics adhesives must be partially accepted, as the

incorporation of TCN-HNT at a concentration higher than 5% interfered with the polymerisation rate of some adhesives.

The HNT filler used in this study is characterised by multi-walled inorganic nanotubes, whose structure is tubular with aluminols in its inner part and siloxanes and silanols/aluminols groups in the outer portion of nanotube [30]. Brackets and archwires usually create numerous retention sites hampering tooth cleaning and increasing potential to develop white spot lesions on enamel around them[31]. Because of this unique composition, the adhesives containing HNTs showed bioactive mineral precipitation on their surfaces after 14 days of immersion in AS. This bio-reactivity may be attributed due exchange of hydrogen ions [H+] with the buffer solution and the SiO_2 layer, which attracts Ca^{2+} and PO_4^{3-} [32]. This may serve as a source of ions to prevent demineralisation of enamel when low pH occurs due to plaque accumulation during orthodontic treatment.

Moreover, the HNT can also serve as a reservoir for sustained release of active substances such as antibacterial agents [33, 34]. In this regard, the neutral charge of triclosan allows its localisation inside and outside of nanotubes [35], as shown in Figure 1. The relatively hydrophobicity of the lumen of HNT favoured the penetration of hydrophobic molecules of triclosan into the nanotubes [11, 33]. With a concentration of triclosan of 20% it was possible to obtain an inhibition effect on the growth of *S. mutans* up to 72 h. The limitation of this study was the antibacterial effect that could not be evaluated due to bacteria death after 72 h. Usually, the antimicrobial effect of materials is assessed at 24 h [36]. Despite the challenged behaviour of this supersaturated model of *S. mutans*, in this study we evaluated antibacterial action up to 72 h to test the persisting

effect of TCN-HNT. Although a decreased of antibacterial activity after 72 h compared to 24 h of incubation for the experimental groups, the prolonged diffusion of triclosan from the nanotubes may have diffused over time into bacterial membrane, improving the antimicrobial capability of the experimental adhesive containing 20% of TCN-HNT [37]. Thus, the second hypothesis of this study must be accepted as different amounts of TCN-HNT incorporated to orthodontic adhesives had different effect on the antibacterial and bioactivity properties of the experimental adhesive tested in this study.

5. CONCLUSION

The present findings confirm that triclosan-doped halloysite nanotubes can be successfully incorporated into resin-based materials up to 20 wt.%. Such therapeutic adhesives may promote long-term antimicrobial activity and mineral deposition, with reliable degree of conversion *in situ* and shear bond strength. Thus, the use of orthodontic adhesives containing triclosan-doped halloysite nanotubes may represent a promising approach to prevent in vivo demineralisation and therapeutic enamel remineralisation during orthodontic treatments. However, such hypothesis must be confirmed by further in vivo and clinical trials studies.

6. REFERENCES

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Table 1. Antimicrobial effect in log_{10}CFU/mL at different incubation time, degree of conversion in situ (DC%), maximum polymerisation rate [Rp.(s-1)] and shear bond strength (MPa) of the adhesive with different concentrations of TCN-HNT.

Groups	Log_{10} CFU/mL			DC *in situ* (%)	Max. polymerisation rate	Shear bond strength (MPa)
	24 h	48 h	72 h			
0%	$9.19 \pm 0.15^{B,b}$	$8.21 \pm 0.29^{B,a}$	$7.97 \pm 0.36^{B,a}$	76.85 ± 1.09^{A}	12.23 ± 4.42^{AB}	17.77 ± 4.70^{A}
5%	$6.99 \pm 1.07^{A,a}$	$8.10 \pm 0.38^{B,b}$	$7.54 \pm 0.12^{AB,b}$	75.69 ± 1.87^{A}	14.46 ± 3.31^{A}	17.23 ± 4.91^{A}
10%	$6.02 \pm 0.87^{A,a}$	$6.95 \pm 0.41^{A,b}$	$7.31 \pm 0.09^{AB,b}$	76.72 ± 3.03^{A}	9.11 ± 0.24^{AB}	13.51 ± 2.93^{A}
20%	$5.42 \pm 1.44^{A,a}$	$7.07 \pm 0.35^{A,b}$	$6.84 \pm 0.41^{A,b}$	74.05 ± 2.42^{A}	7.70 ± 0.83^{B}	13.80 ± 2.22^{A}

Different uppercase letters in the same column means statistically significant difference ($p < 0.05$).
Different lowercase letters in the same row means statistically significant difference ($p < 0.05$).

FIGURES CAPTIONS

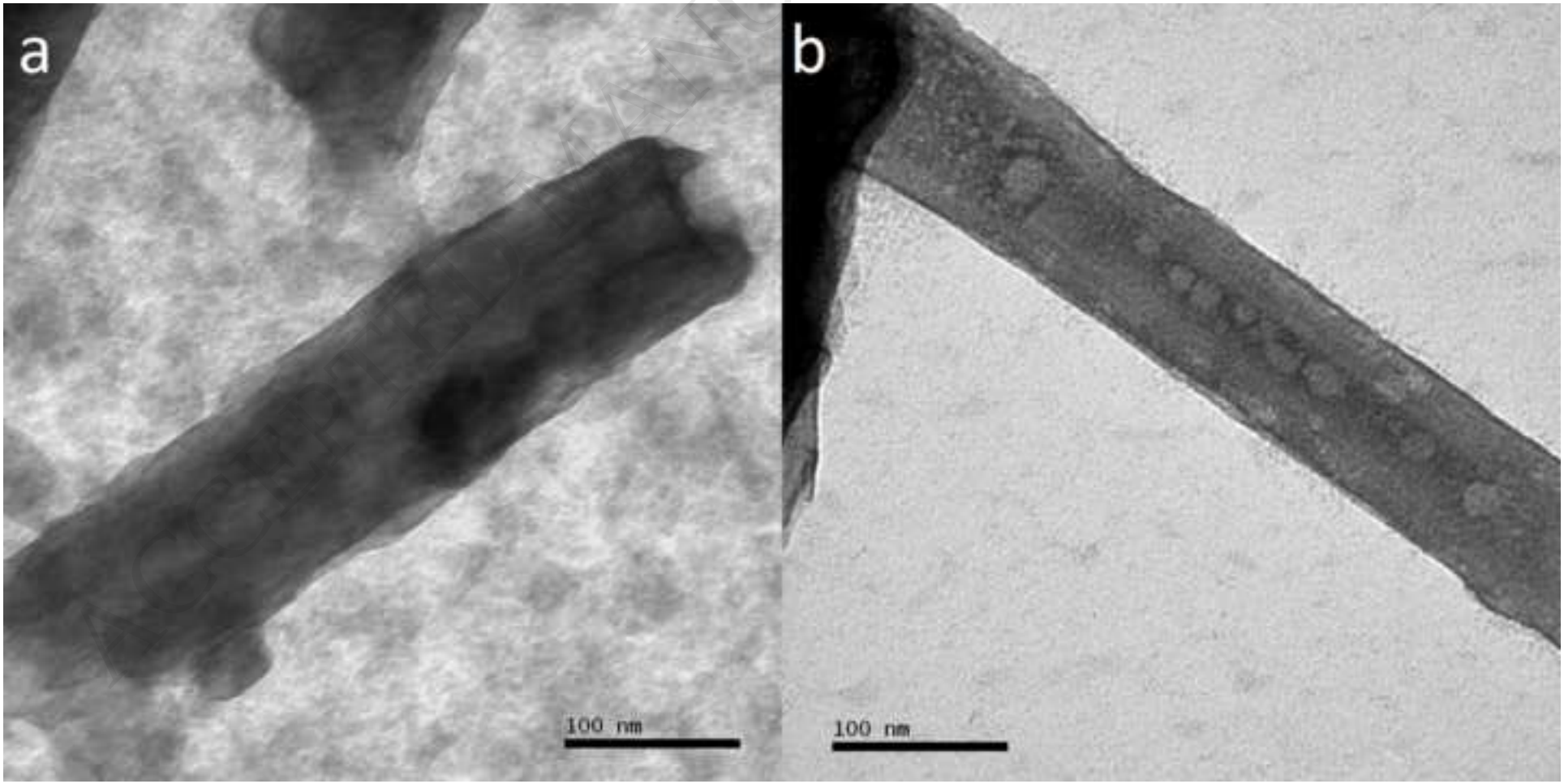
Figure 1. (a) TEM image of a nanotube with its inner-surface of 40–50 nm diameter range and outer-surface length of 90 nm diameter. (b) TEM image show the presence of TCN nanoparticles inside nanotubes. It is also possible to observe empty dark spaces, which may permit the mobility of triclosan through the tube.

Figure 2. ARI score of the adhesive with different concentrations of TCN-HNT. Different letters mean statistical difference between groups ($p<.05$).

Figure 3. SEM images with EDS scales of mineral deposition. In images a, b, c and d, no mineral deposition was observed prior artificial saliva. e) No mineral deposition after saliva immersion in control group. In images f, g and h, mineral deposition was observed in adhesives containing respectively 5%, 10% and 20% of TCN-HNT after 14 days of saliva immersion signalized by an orange target and confirmed by EDS scales. Dark line highlights interface between adhesive (A) and enamel (E) areas.

Figure 4. Analyses of interface between adhesive and enamel surface with different TCN-HNT concentrations, initially and after 14 days. Arrows indicate mineral deposition on interface with adhesive containing 5%, 10% and 20% of TCN-HNT after 14 days of immersion in artificial saliva. Peaks of 960 cm^{-1} and 1610 cm^{-1} represent calcium and methacrylate chemical groups, respectively. Crossing lines represent interface, E represents enamel side and A represents adhesive side.

Figure



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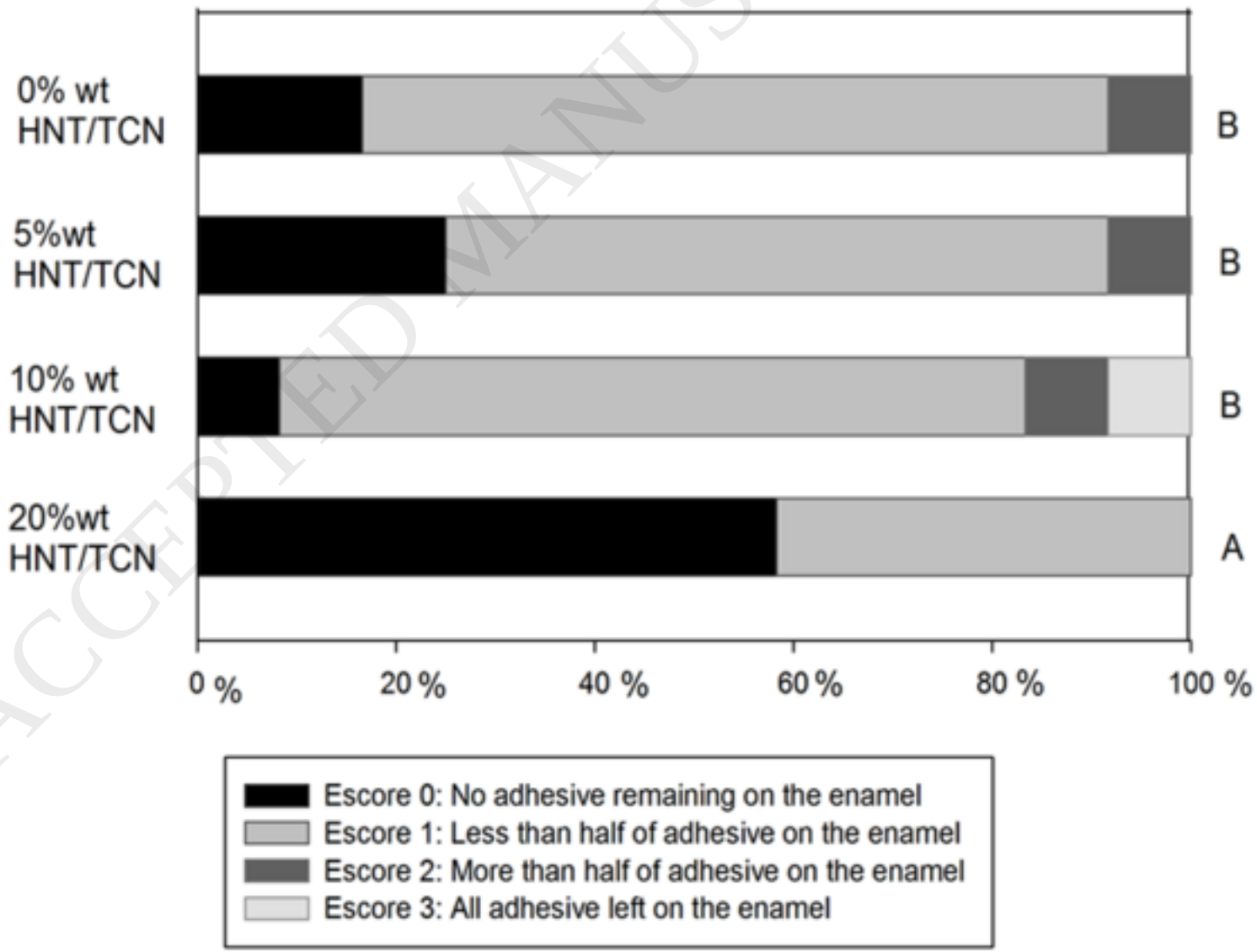

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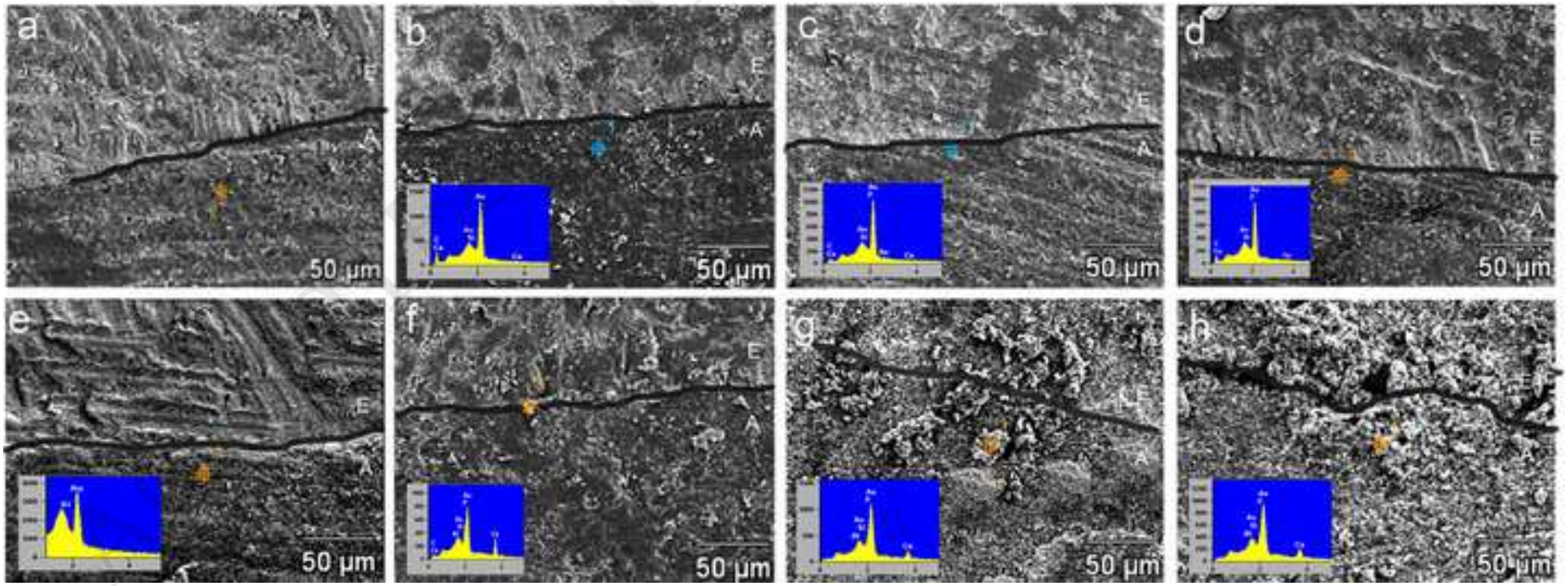

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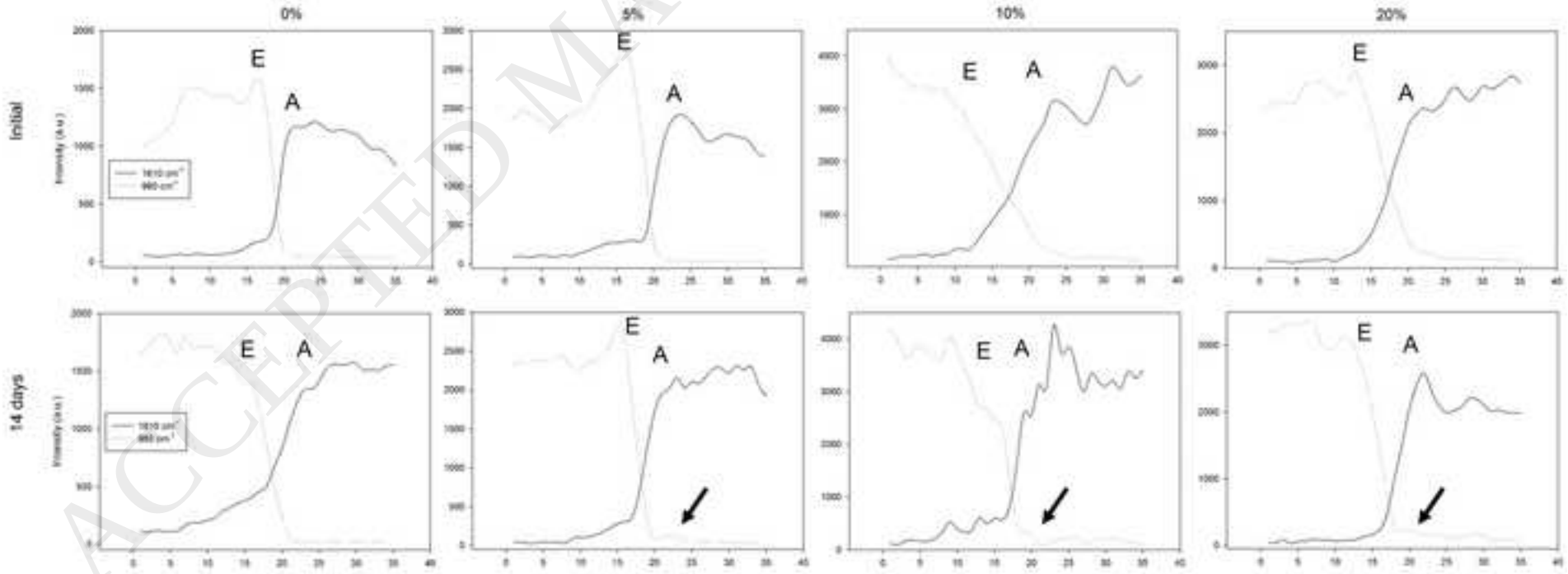

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To The Editor of

Journal of Dentistry **25/07/2017**

Dear Professor Dr. Christopher D. Lynch

PERMISSION NOTE

Herewith the permission letter for our original article **"Polymerisation, antibacterial and bioactivity properties of experimental orthodontic adhesives containing triclosan-loaded halloysite nanotubes**" submitted to Journal of Dentistry.

Thank very you much

Yours Sincerely,

Prof. Dr. Salvatore Sauro

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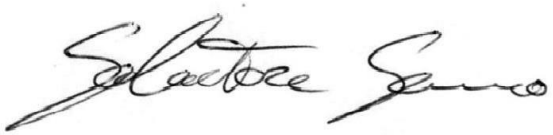

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All authors have contributed significantly, and they are all in agreement with the manuscript. They gave full permission to publish photographs in all forms and media

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We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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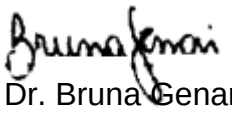
Dr. Felipe Weidenbach Degrazia



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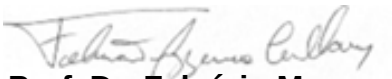

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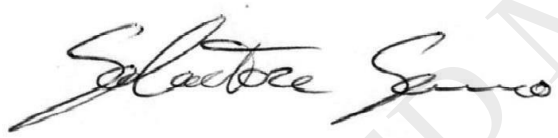

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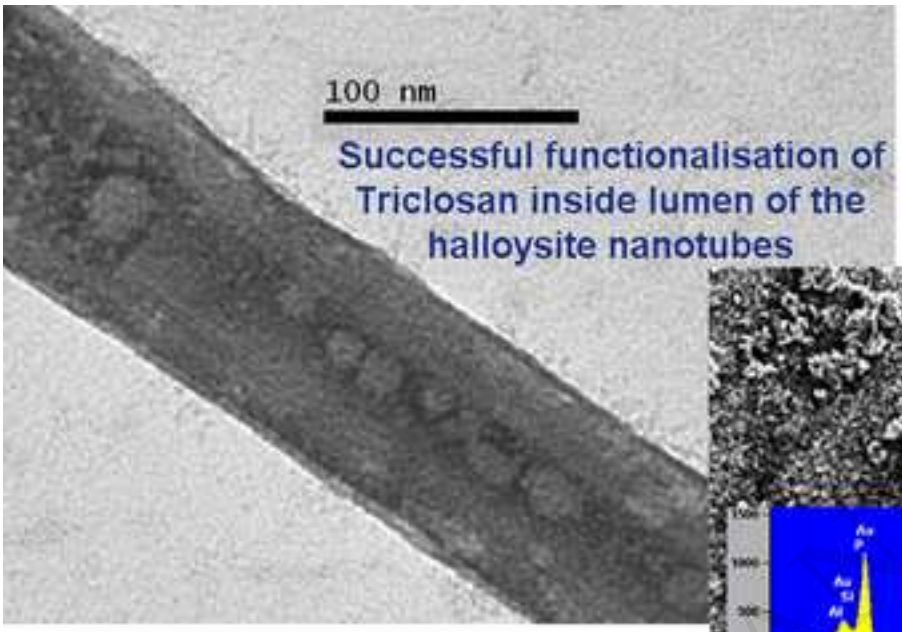
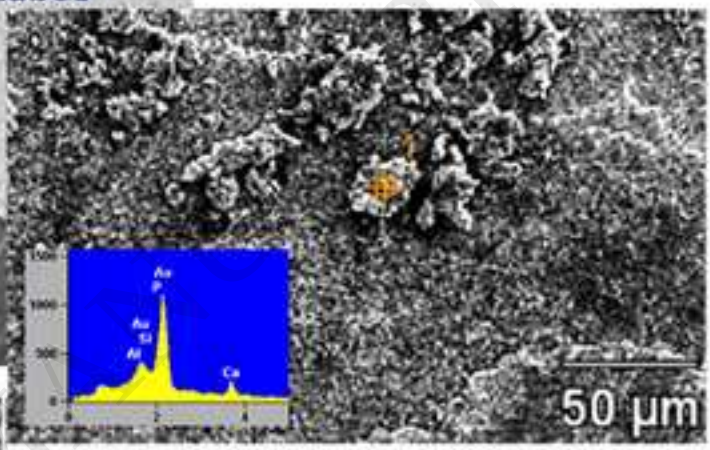
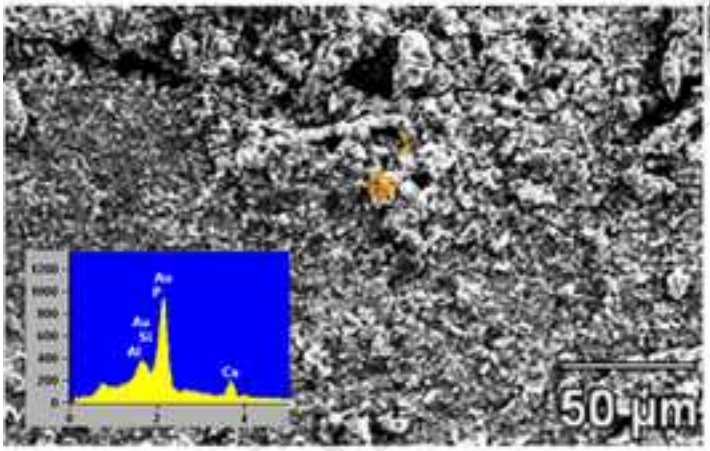
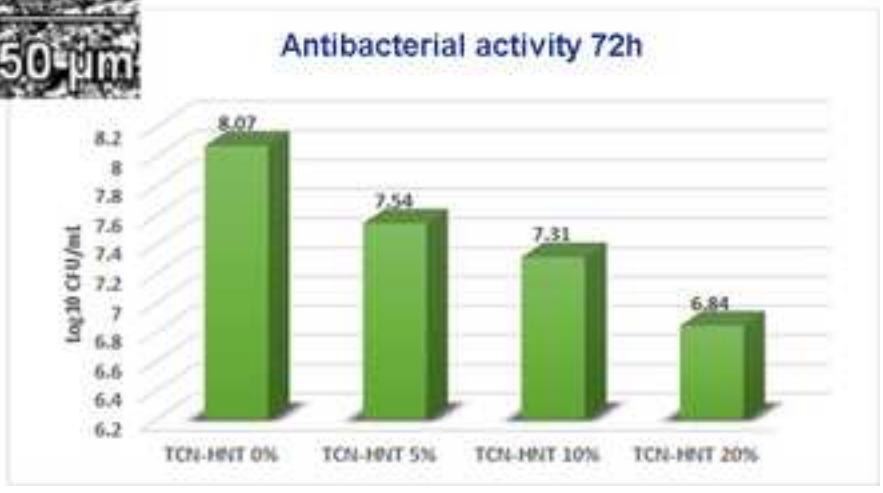






TCN-HNT induces mineral precipitation "Bioactivity".



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